Pharmacologic Evidence for Involvement of Phospholipase C in Pemphigus IgG–Induced Inositol 1,4,5-Trisphosphate Generation, Intracellular Calcium Increase, and Plasminogen Activator Secretion in DJM-1 Cells, a Squamous Cell Carcinoma Line

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The precise mechanism for acantholysis after pemphigus IgG binds to the cell surface is as yet unknown, although involvement of proteinases such as plasminogen activator (PA) has been suggested. We previously reported that pemphigus IgG, but not normal nor bullous pemphigoid IgGs, caused a transient increase in intracellular calcium ($[Ca^{++}]_i$) and inositol 1,4,5-trisphosphate (IP_3) concentration in cultured DJM-1 cells (a squamous cell carcinoma line). To clarify whether phospholipase C is involved in this process after the antibody binds to the cell surface, we examined the effects of a specific phospholipase C inhibitor (U73122) on the pemphigus IgG–induced increase in $[Ca^{++}]_i$, IP_3, PA secretion, and cell-cell detachment in DJM-1 cells.

$[Ca^{++}]_i$ and IP_3 contents were determined with or without 30-min pre-incubation with U73122 or an inactive analogue (U73343) with fura-2 acetoxymethylester and a specific IP_3 binding protein, respectively. PA activity in the culture medium was measured after various incubation periods with pemphigus IgG by two-step amidolytic assay. The detachment of cell-cell contacts was examined by detecting the retraction of keratin filament bundle from cell-cell contact points to the perinuclear region by immunofluorescence microscopy using anti-keratin antibody. Pemphigus IgG immediately increased $[Ca^{++}]_i$ and IP_3 content. PA activity in the culture medium has also been increased at 24 h after pemphigus IgG was added in association with cell-cell detachment. However, pre-incubation with U73122 (1–10 μM), but not with U73343 (10 μM), dramatically reduced the pemphigus IgG–induced increases in $[Ca^{++}]_i$, IP_3, and PA activity and inhibited the pemphigus IgG–induced cell-cell detachment. Both U73122 and U73343 caused no effects on cell viability and IgG binding to the cell surface. These results suggest that phospholipase C plays an important role in transmembrane signaling leading to cell-cell detachment exerted by pemphigus IgG binding to the cell surface. *Key words: bullous diseases/keratinocyte/signal transduction/calcium. J Invest Dermatol 105:329–333, 1995*

Pemphigus vulgaris (PV) is characterized by the autoantibody against desmoglein III [1–3], which forms a 210-kD protein complex with plakoglobin [4] and is considered to be a desmosomal protein [1,5]. It has been suggested that pemphigus-induced acantholysis is profoundly related to proteinase secretion from keratinocytes [6] such as secretion of plasminogen activator (PA) [7]. However, the precise mechanism for acantholysis after pemphigus-IgG binds to the cell surface is as yet unknown. We recently reported that pemphigus IgG induced a transient increase of intracellular calcium concentration ($[Ca^{++}]_i$) and inositol 1,4,5-trisphosphate (IP_3), although bullous pemphigoid (BP) IgG did not [8]. In this regard, it is of great interest to note that pemphigus IgG did not inhibit the Ca^{++} switch–induced desmosome formation of low-Ca^{++}–cultured keratinocytes, which lacked desmosomes [9], whereas BP-IgG disturbed the Ca^{++} switch–induced reassembling of hemidesmosome components on the bottom cell surface by internalization of the immune complex of BP-IgG and 180-kD BP antigen [10,11]. These results suggest that there must be an intrinsic difference in the cell-biologic response of keratinocytes to the pathogenic antibodies between pemphigus and pemphigoid. From these viewpoints, it appears to be important to study the precise cell responses after the binding of the antibodies to the surface antigen focusing on the antibody-induced transmembrane signaling for the mechanisms of blistering in pemphigus and pemphigoid.

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Abbreviations: BP, bullous pemphigoid; $[Ca^{++}]_i$, intracellular calcium concentration; IP_3, inositol 1,4,5-trisphosphate; PA, plasminogen activator; PI, phosphoinositide; PV, pemphigus vulgaris; U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; U73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione.

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Phosphoinositide (PI) turnover has been shown to play an important role in the regulation of cellular growth and differentiation [11]. Growth factors and extracellular signals cause hydrolysis of phosphatidylinositol 4,5-bisphosphate through receptor-mediated activation of PI-specific phospholipase C (PI-PLC). Phosphatidylinositol 4,5-bisphosphate hydrolysis generates two messengers: IP_3, which induces Ca^{++} mobilization from intracellular stores, and 1,2-diacylglycerol, which activates protein kinase C [12–14]. Upon pemphigus IgG binding to the cell surface, the mass contents of IP_3 and the $[Ca^{++}]_i$ were markedly increased, peaking at 20–30 seconds, indicating a possibility that PI-PLC is activated as a trigger for transmembrane signaling after the binding. Therefore, in the present experiments, we studied the effects of co-incubation with a specific inhibitor of PLC (U73122) and its inactive analogue, U73343 [15–19] on the PV IgG–induced changes of $[Ca^{++}]_i$, IP_3, PA secretion, and cell-cell detachment to clarify whether or not PLC is involved in keratinocyte responses to pemphigus antibody.

MATERIALS AND METHODS

Reagents The quantitative assay kit for D-myo inositol 1,4,5-trisphosphate (IP_3) was obtained from Amersham (Buckinghamshire, UK). Fura-2 acetoxymethylester (fura-2/AM) was purchased from Dojin Laboratories (Kumamoto, Japan). The pharmacologic agents U73122 (1-[6-[[17 β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) and U73343 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione) were from Biomol Research Lab (Plymouth, PA). Plasminogen from human plasma was from Boehringer Mannheim GmbH, Mannheim, Germany. All other chemicals were of reagent grade.

Sera Sera from a PV and a BP patient, and a normal volunteer without any skin diseases were used for experiments. By immunoblotting with ethylenediaminetetraacetic acid–separated normal human epidermal extract and the extract from DJM-1 cells, antibodies against 130-kD PV antigen and 180-kD BP antigen were detected in these PV and BP sera, respectively. Both sera were stored at −80°C. IgG fractions were isolated from these sera by HiTrap protein A affinity column (Pharmacia AB, Uppsala, Sweden). The purity of isolated IgG was checked by immunoelectrophoresis. The specificities of the PV-IgG for binding activity to 130-kD peptide on the keratinocyte cell surface, for the IP_3 production, and for $[Ca^{++}]_i$ increase were confirmed in our previous paper [8].

Cell Culture An isolated cell line (DJM-1) from human skin squamous cell carcinoma [9,20,21] was cultured in 1.8 mM Ca^{++} medium containing Eagle's minimum essential medium (MEM), 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 units/ml penicillin at 37°C in humidified 5% CO_2/95% air. The medium was changed every 2 d. On reaching confluence, the cells were treated with Hanks' balanced salt solution containing 0.02% trypsin and 0.02% ethylenediaminetetraacetic acid for 1 h at 37°C. The cells were resuspended in MEM with 10% FBS, and cultured for 2 d, and then used for the following experiments. Cell viability was checked by staining with trypan blue.

Immunofluorescence Studies of the Effects of U73122 on PV-IgG–Induced Cell-Cell Detachment To check the binding activities of antibody to the cell surface of keratinocytes, indirect immunofluorescence microscopy was performed by standard methods using normal human skin section and cultured DJM-1 cells as substrates [9].

The effects of PV antibody to the cell-cell contact of DJM-1 cells were studied by examining the cytoskeletal organization of keratin intermediate filaments by immunofluorescence microscopy. Antikeratin monoclonal antibody, LP34 (DAKO Corp., Carpinteria, CA) that reacted with human cytokeratin 18 and 6 (molecular weight 45 kD and 56 kD, respectively) was employed as the first antibody. To examine the effects of U73122 on the pemphigus antibody–induced detachment of cell-to-cell contacts, DJM-1 cells were incubated in the medium containing 10% pemphigus serum with U73122 (10 μM) or 10% pemphigus serum alone, and then subjected to immunostaining for keratin intermediate filaments.

Measurement of Intracellular Calcium ($[Ca^{++}]_i$) in Cultured Cells [22,23] Cells were loaded with calcium indicator fura-2/AM (5 μM) by incubation for 2 h at room temperature in MEM. After pre-incubation with or without U73122 or U73343 for different times, the cells were washed twice with MEM and exposed to IgGs (1 mg/ml) diluted with Hanks' balanced salt solution at 37°C. Fluorescence images were obtained at alternating excitation wavelengths of 340 and 360 nm through a SIT vidicon camera, and were processed by an ARGUS-100 image analyzer (Hamamatsu Photonics Corp., Hamamatsu, Japan). The calibration of the fluorescent signal in terms of $[Ca^{++}]_i$ was performed as previously described [23].

Inositol 1,4,5-Trisphosphate Measurement The assay for IP_3 was performed using the quantitative assay kit for D-myo IP_3 (Amersham, Buckinghamshire, UK) as described previously [8,24]. For quantitation of IP_3 levels, the cells in 24-well culture trays were pre-incubated with or without U73122 or U73343 at 37°C for 30 min, and then the medium was quickly aspirated and IgGs (1 mg/ml) diluted with Hanks' balanced salt solution containing appropriate concentrations of U73122 or U73343 were added. The reaction was terminated at different intervals by adding 10% ice-cold perchloric acid, and the reaction mixture was then neutralized by the addition of 1.53 M KOH containing 75 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The supernatant of this reaction mixture was added to IP_3 assay mixture containing a specific IP_3-binding protein (Amersham) and was incubated for 15 min at 4°C prior to centrifugation at 2000 × *g* for 15 min. The resulting pellets were resuspended in water and measured for radioactivity. The amount of IP_3 was determined from a standard curve using authentic IP_3.

PA Activity Assay DJM-1 cells (2×10^6 cells/dish) were seeded in 60-mm dishes and grown in MEM with 10% FBS for 2 d until subconfluent. Then, the dishes were rinsed three times with MEM without FBS, followed by incubation with MEM (3 ml) containing IgG (1 mg/ml) from PV, BP, or normal sera for 9, 15, 24, or 36 h. In addition, different concentrations of U73122 were added in the culture medium with PV IgG. Cell-culture supernatants (approximately 3 ml) were removed at different times, and stored at −80°C. Then, a two-step amidolytic assay was used to measure PA activity [25,26]. This assay was based on the conversion of plasminogen to plasmin that catalyzes the splitting of p-nitroanilline (pNA) from the chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2251; Kabi Pharmacia Hepar Inc., Franklin, OH). The released pNA is measured photometrically at 405 nm using the end-point method. Briefly, 50 μl of buffer (50 mM Tris, 12 mM NaCl, pH 7.4 at 25°C) containing 30 μg/ml of plasminogen was allowed to equilibrate at 37°C for 5 min, followed by addition of 25 μl of sample and incubation at 37°C for 10 min. Reaction was started by addition of 175 μl of S-2251 (0.48 mg/ml), and then after 15 min stopped by adding 25 μl of 20% acetic acid. PA readings were blanked against the culture medium. Using purified human urokinase as a standard, the assay was determined to be linear over the range 0.005–0.0001 IU/μl. PA activity was shown as IU/ml based on standard curve.

Data Analysis Statistical analysis of the data was carried out by Student t test.

RESULTS

No Effects of U73122 on Cell Viability and IgG Binding to the Cell Surface At the outset, non-specific effects of the pharmacologic agents used here, which were a specific PLC inhibitor, U73122; its inactive analogue, U73343; and a vehicle, dimethyl sulfoxide (DMSO), on the cell viability and binding activity of PV-IgG to the cell surface of DJM-1 cells were checked. No significant difference in the viabilities (trypan blue exclusion test) was detected among the cells incubated with 1 and 10 μM of U73122, 10 μM of U73343, and 0.01% DMSO for 30 min, revealing 91.5 ± 3.9%, 93.7 ± 1.3%, 88.4 ± 2.1%, and 92.1 ± 3.5% viability, respectively. No morphologic changes suggesting cell death or degeneration were observed in the cells during appropriate experimental periods after incubation with these reagents. No effects of U73122 and U73343 were exerted on the PV-IgG binding activity to the cell surface after the cells were incubated with these agents at the concentration of 10 μM for 30 min when the cells were examined by immunofluorescence using anti-human IgG conjugated with fluorescein isothiocyanate **(Fig 1*a,b*)**.

Inhibition of PV-IgG–Induced $[Ca^{++}]_i$ Response by U73122 The resting $[Ca^{++}]_i$ estimated from the fluorescence ratio (F340/F360) was 103.3 ± 37.0 nM (n = 160). The addition of PV-IgG resulted in a rapid and transient $[Ca^{++}]_i$ increase, peaking 20 seconds after stimulation, followed by a gradual return to the basal level in DJM-1 cells **(Fig 2*a*)**. The pre-incubation with 1 μM **(Fig 2*c*)** or 10 μM **(Fig 2*d*)** of a specific inhibitor of PLC, U73122, for 30 min or 10 μM for 5 min **(Fig 2*e*)** reduced markedly the

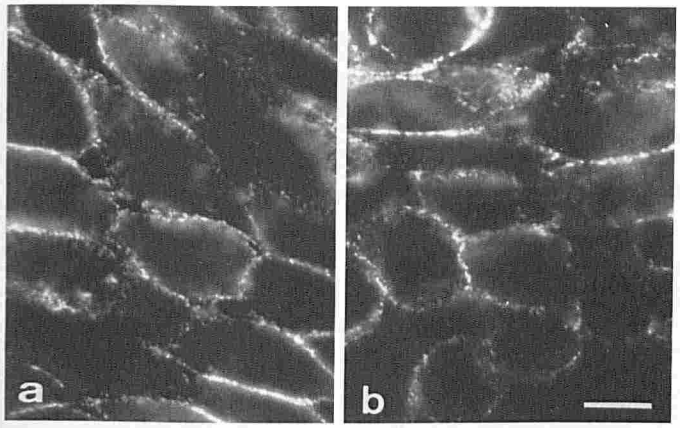

Figure 1. Indirect immunofluorescence study showing no effect of U73122 and U73343 on PV-IgG binding on the cell surface. Indirect immunofluorescence using PV-IgG after incubation with 10 μM of U73122 (*a*) and U73343 (*b*) for 30 min shows that these agents did not affect the activity of PV-IgG binding on the cell surface of DJM-1 cells. *Bar*, 10 μM.

PV-IgG–induced $[Ca^{++}]_i$ increase. On the other hand, the $[Ca^{++}]_i$ increase induced by PV-IgG was not affected by pre-incubation with MEM containing 0.01% DMSO **(Fig 2*b*)** or 10 μM of an inactive analogue of U73122, U73343 **(Fig 2*f*)** for 30 min.

Inhibition of PV-IgG–Induced IP_3 Generation by U73122 The mass content of IP_3, which is known to induce the calcium mobilization from internal calcium store(s), was measured in DJM-1 cells exposed to PV-IgG. Because PV-IgG elicits a rapid and transient production of IP_3 peaking at 20 seconds [8], the mass contents of IP_3 were measured at 20 seconds after the addition of PV-IgG. Pre-incubation with U73122 (1–100 μM) for 30 min inhibited the PV-IgG–induced IP_3 production, whereas U73343 (10 μM) or 0.01% DMSO, which was a vehicle, did not affect the IP_3 generation induced by PV-IgG **(Fig 3)**. No IP_3 generation by PV-IgG was detected throughout incubation periods (until 5 min) after IgG addition, when the PLC inhibitor U73122 was added in the culture medium.

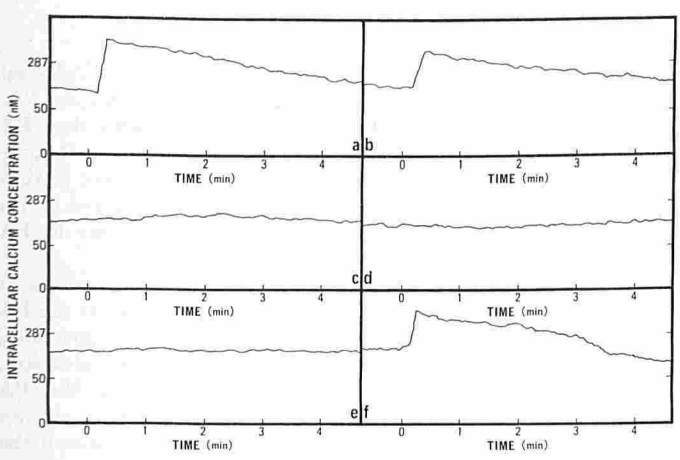

Figure 2. Inhibition of PV-IgG–induced $[Ca^{++}]_i$ increase by U73122. Representative time courses are shown of PV-IgG–induced $[Ca^{++}]_i$ response in DJM-1 cells loaded with fura 2/AM. The $[Ca^{++}]_i$ responses to PV-IgG after the preincubation with MEM (*a*), 0.01% DMSO (*b*), 1 μM (*c*), or 10 μM (*d*) of U73122, or 10 μM of U73343 (inactive isoform of U73122) (*f*) for 30 min were shown. In addition, *e* shows the $[Ca^{++}]_i$ response to PV-IgG after incubation for 5 min. U73122 (1 and 10 μM), but neither vehicle nor U73343, inhibited the PV-IgG–induced $[Ca^{++}]_i$ increase.

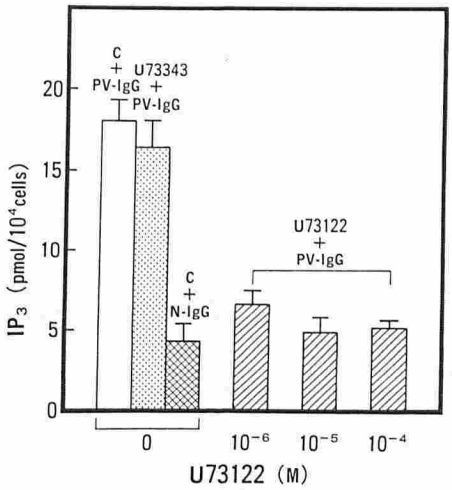

Figure 3. Reduction of PV-IgG–induced IP_3 production by U73122. The mass contents of IP_3 in DJM-1 cells at 20 seconds after the addition of PV-IgG were shown. Cells were preexposed to vehicle (0.01% DMSO) (C + PV-IgG); 1, 10, or 100 μM of U73122 (U73122 + PV-IgG); or 10 μM of U73343 (U73343 + PV-IgG) for 30 min, and PV-IgG was then added to the culture medium. U73122 (1–100 μM) prominently reduced IP_3 generation induced by PV-IgG ($p < 0.001$) compared to the vehicle control (C + PV-IgG), whereas U73343 did not. The content of IP_3 at 20 seconds after the addition of normal human IgG to DJM-1 cells (C + N-IgG) shows no increase at the basal level.

Inhibition of PV-IgG–Induced Secretion of Plasminogen Activator by U73122 The activity of PA secreted into culture medium was increased in time-dependent fashion until 24 h and then reached the plateau, in the case of all additions to the medium. PV-IgG remarkably enhanced the PA activity compared to BP-IgG and normal-IgG at 24 and 36 h ($p < 0.005$). U73122 reduced dramatically the PV-IgG–induced increase of activity of PA in cultured medium to the lowest levels of PA activities induced by BP and normal IgGs. The differences in PA activity at 24 and 36 h between the results with and without U73122 (10^{-4} to 10^{-6} M) were statistically significant ($p < 0.005$). In contrast, no significant difference was observed in the PV-IgG–induced increase of PA activity in culture medium between with or without U73343 (10^{-5} M) **(Fig 4)**. In addition, U73122 (10^{-5} M) reduced the enhanced PA activity induced by IgGs from two more PV sera at 24 and 36 h.

Inhibition of PV-IgG–Induced Detachment of Cell-Cell Contacts by U73122 We have previously shown that PV-IgG causes the detachment of cell-cell contacts in DJM-1 cells, as visualized by retraction of keratin intermediate filaments by immunofluorescence using anti-keratin antibodies [9]. In the present experiments, we checked whether or not a pharmacologic agent, U73122, which specifically inhibits PLC activity, could abrogate the PV-IgG–induced cell-cell detachment. Pre- and co-incubation of U73122 (10 μM) with PV serum (10%) inhibited the antibody-dependent retraction of keratin intermediate filaments, which was caused by a 24-h incubation with 10% PV serum in the control experiments, as shown in **Fig 5*a,b***. In addition, when purified PV-IgG was used instead of PV serum, the same results were obtained as with PV serum, as shown in **Fig 5**.

DISCUSSION

Although mechanism of acantholysis induced by pemphigus antibody is still unknown, the activation of proteinases has been suggested to play a role [6]. In addition, it was demonstrated that the binding of antibodies to the cell surface resulted in the release of PA [7], so that the plasmin system could be activated and possibly induce acantholysis [27]. Because it is likely that PV-IgG triggers the secretion of PA by cultured keratinocytes as mentioned above,

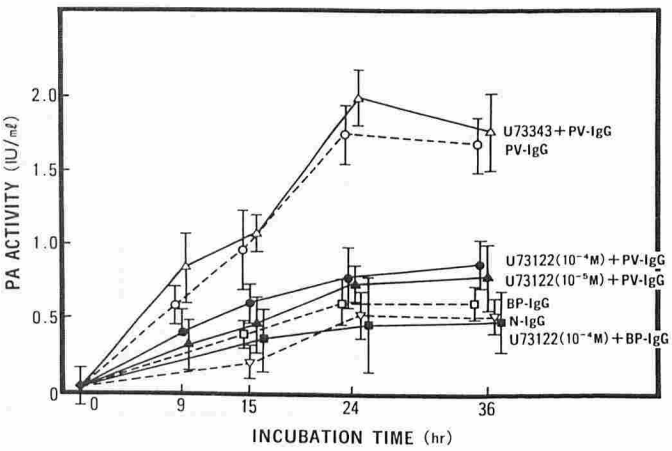

Figure 4. Inhibition of PV-IgG–induced PA secretion by U73122. The addition of PV-IgG, BP-IgG, or N-IgG to the culture medium caused some increase in the activity of PA in the culture medium of DJM-1 cells, which is accelerated during the incubation. PA activities were shown as IU/ml based on a standard curve. PV-IgG distinctly enhanced the PA activity compared to BP or N-IgGs at 24 and 36 h ($p < 0.005$). The activities of PA secreted in the culture medium mixed with PV-IgG + 0.01% DMSO as a control vehicle for inhibitors (PV-IgG), PV-IgG plus different concentrations of U73122 (U73122 + PV-IgG), or PV-IgG plus 10 μM of U73343 (U73343 + PV-IgG) are shown for indicated intervals. PV-IgG–induced PA secretion was dramatically decreased by U73122 (10–100 μM) compared to the vehicle control (PV-IgG) and the inactive analogue control (U73343 + PV-IgG) after 24 and 36 h ($p < 0.005$).

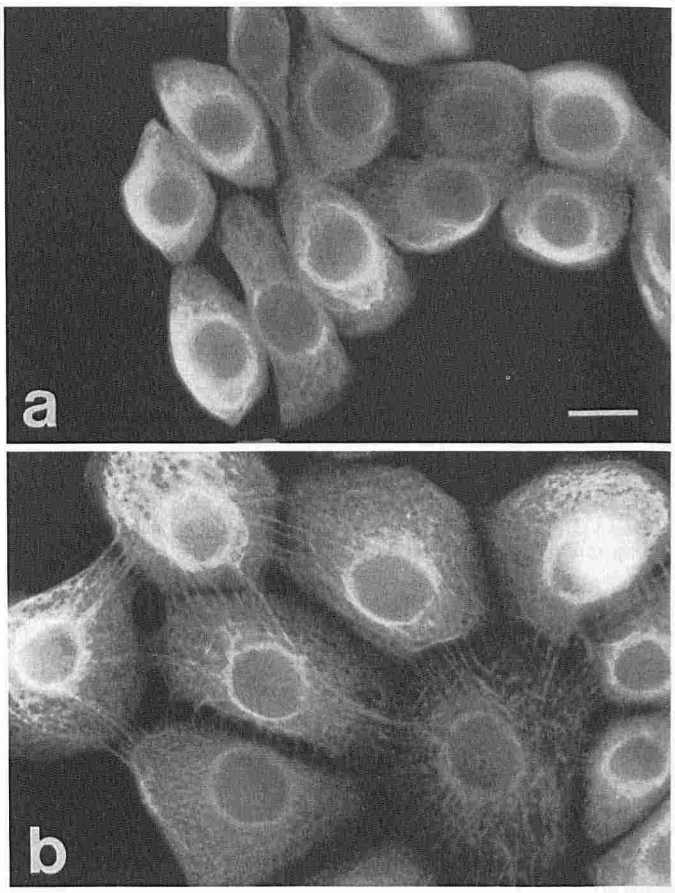

Figure 5. Inhibition of PV-serum–induced detachment of cell-cell contacts by U73122. After the incubation with 10% PV serum (treated at 56°C for 30 min) containing vehicle (*a*) or 10 μM of U73122 (*b*) for 24 h, DJM-1 cells were stained with LP34, anti-keratin antibody, which reacted with human cytokeratin 18 and 6. The PV-serum–induced retraction of keratin filaments from the cell periphery to the perinuclear region was observed without U73122 (*a*), whereas this change was inhibited and the keratin bundle arrangements were clearly observed to extend to the cell-cell contacts in the presence of U73122 (*b*). *Bar*, 10 μM.

it appears reasonable to speculate that the binding of PV-IgG to the cell-surface antigen desmoglein III may transmit a transmembrane signal into the cell and result in secretion of PA. Therefore, we previously studied the Ca^{++}-mediated signaling pathway activated by PV-IgG and showed that PV-IgG caused a rapid and transient increase of IP_3 and intracellular Ca^{++}, which we suggested was mobilized from internal stores [8]. In this later study we examined the involvement of PLC in the increases of intracellular IP_3 and Ca^{2+} concentration and the secretion of PA generated by the binding of PV-IgG to the cell surface.

We employed a pharmacologic experiment using a specific inhibitor of PLC, U73122, and its inactive analogue, U73343, to examine the role of PLC in PV-IgG–induced generation of IP_3, which in turn leads to a rapid increase in intracellular Ca^{++} concentration. We found that a specific PLC inhibitor, U73122, dramatically reduced the transient increase of IP_3 and $[Ca^{++}]_i$ caused by PV-IgG without exerting any effects on cell viability or binding activity of PV-IgG to the cell surface. Furthermore, we confirmed the enhancement of PA secretion by PV-IgG in cultured keratinocytes and found that the PLC inhibitor markedly inhibited PV-IgG enhancement of the PA secretion from keratinocytes in our experimental systems of DJM-1 cells. These results suggest that PLC is involved in the generation of IP_3, mobilization of Ca^{++}, and finally secretion of PA triggered by PV-IgG.

The activity of PA is known to be increased in several other skin diseases including psoriasis [28], which is not associated with the loss of cell to cell contacts. Also, it has been shown that dexamethasone inhibits PA activity in pemphigus but does not block acantholysis [29]. However, our study showed that inhibition of PLC by U73122 dramatically reduced the secretion of PA **(Fig 4)** in association with inhibition of the PV-IgG–dependent detachment of cell-cell contacts **(Fig 5)**, as detected by immunofluorescence study of the retraction of keratin intermediate filaments. In addition, we have previously shown that PV-IgG did not inhibit directly the Ca^{++}-switch–induced desmosome formation [9], whereas BP-IgG inhibited the Ca^{++}-switch–induced reassembling of hemidesmosome components at the basal surface of cultured keratinocyte in the same experimental system [10,20]. This suggests that PV-IgG has no detectable activity as a blocking antibody to cell-cell contacts, so that digestion of cell-cell contacts, for example, by PA, may be more important. Therefore, it appears feasible that PA secretion has some relevance to the PV-IgG–dependent detachment of cell-cell contacts, so that we have to carry out more studies along this line in the future to understand why acantholysis is not induced in other skin diseases associated with an increase in PA activity.

Whether or not the increased PA activity might be involved in acantholysis in pemphigus, it is likely that PV-IgG enhances the PA secretion, and this enhancement of PA secretion is apparently mediated *via* PLC-activated Ca^{++}-dependent signal transduction. Involvement of some other factors, however, such as the PA receptor and PA inhibitor in mechanisms for acantholysis, cannot be excluded. It is of interest that another mechanism in which the binding of antibodies to the cell surface induced a conformational change of intracellular proteins, probably due to phosphorylation, was suggested by the finding that pre-incubation with the protein kinase C inhibitor H7 prevented the PV serum-induced dissociation of cell-to-cell contact [30]. In this regard, protein kinase C is known to be activated with diacylglycerol produced by PLC activation [12–14].

The characteristics of action of an aminosteroid inhibitor, U73122, on PLC-mediated events were described for neuroblas-

toma cells [17] and pituitary cells [18]. Although the precise site of action of U73122 is unclear, these previous works suggest that it probably acts at an early stage in the transduction mechanism and possibly at the level of the G protein Gq [15,16,19,31,32] or on the link between the G protein and the effector. Therefore, certain G proteins might be involved in the regulation of the signal-transduction pathway after binding of the antibody to the cell surface in pemphigus. Furthermore, U73122 has been shown to specifically inhibit all PLC isomers, although it may have some activity toward other phospholipases [33]. Therefore, although the involvement of PLC in PV-IgG–induced responses of DJM-1 cells is suggested to be probable by pharmacologic experiments with U73122 because of its ability to inhibit IP_3 generation, the involvement of other phospholipases cannot be absolutely excluded.

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